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Hyperreactivity of adenines and conformational flexibility of a translational repression site

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We have used a diethylpyrocarbonate (DEPC) modification [(1976) Prog. Nucl. Acids Res. 16, 189–262] to probe the accessibility of adenines essential for coat protein binding in the MS2 translational operator [(1983) Biochemistry 22, 2601–2610, 2610–2615, 4723–4730; (1987) Biochemistry 26, 1563–1568]. The essential adenines are apparently hyperreactive with this reagent relative to other sites within the same molecule. Variation of ionic strength, pH and divalent cation concentrations reveal the existence of two distinct conformers of the RNA operator as judged by DEPC reactivity. We propose that the hyperreactivity observed is due to the participation of neighbouring bases in the DEPC modification reaction and can be used as a novel structural probe.

Diethyl pyrocarbonate; Adenine; Hyperreactivity; Structural probe

1. INTRODUCTION

Many important cellular functions depend on the specific recognition of RNA molecules by proteins. However, our knowledge of such interactions at a molecular level has been limited by a lack of structural information on the RNA components. We have recently developed a modeling algorithm, which can be used to generate three-dimensional structures of simple features of RNA's, such as stem-loop structures [6]. The algorithm uses the structural information in the Brookhaven database of tRNA molecules to produce an initial model which conforms as far as possible with known RNA structures. This can then be 'improved' by energy calculations and tested experimentally by structural probes of the RNA in solution. Here we present our initial results of the application of these techniques to an important and well characterised example of an RNA-protein complex.

The translational repression complex formed between RNA bacteriophage (MS2, R17) coat protein and its RNA is one of the most extensively studied examples of an RNA-protein complex [2–5]. The complex also appears to function as an assembly initiation complex, similar to that described for a simple RNA plant virus [7]. In a very elegant series of experiments Uhlenbeck and his colleagues have demonstrated that a fragment of only 19 bases contains all the structural information necessary to bind protein specifically, and, by using sequence variants they have shown that essentially all the sequence can be varied without affecting binding. The only requirements appear to be for the secondary structure potential to be preserved i.e. the base-pairing of the stem, and for positions −10 to be purine, preferably adenine, −5 to be pyrimidine, with adenine at −7 and −4 (Fig. 1).

In order to examine these requirements we have constructed a three-dimensional graphics model of the RNA stem-loop using a PS300 terminal and the software package FRODO [8] (Fig. 1). The coordinates for the model were taken, as far as possible, from the known X-ray structure of $tRNA^{Asp}$. However, two regions required ab initio modeling, namely the A at −10 and the four-base loop. Details of the modeling have been published elsewhere [6]. However, in summary, these regions were built with a view to maximise (a) hydrogen bonding and (b) base stacking. The modeling suggested that the A at −10 would prefer to intercalate between the surrounding base-pairs and that the loop A's might be able to form a non-Watson-Crick base-pair.

Such intercalation and base-pairing would be expected to alter the surface accessibility of the residues involved and we have therefore tested the accessibilities by chemical modification with diethyl pyrocarbonate (DEPC) [1], a reagent commonly used to identify adenines not participating in Watson–Crick base-pairing. Modified adenines result in strand cleavage on treatment with aniline [9] and the use of end-labelled RNA fragments enables the extent of reaction at particular sites to be determined.

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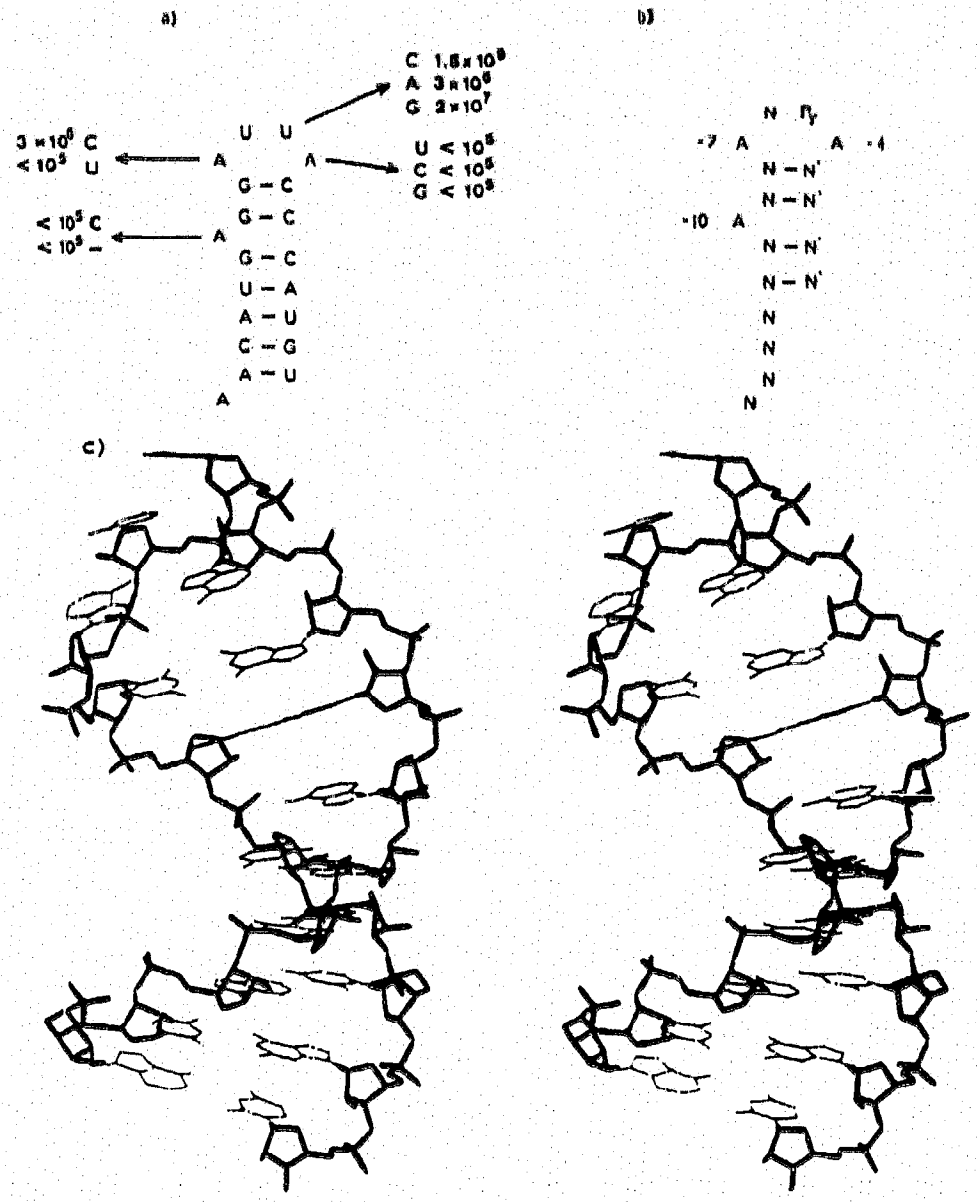

Fig. 1. (a) Proposed secondary structure of the stem-loop fragment of MS2 RNA which contains all the information to bind protein specifically and (b) the minimum information required for specific binding [2,3]. The figures in (a) indicate the values of K_d determined for the sequence changes indicated. (Wild type K_d = 3 × 10^8M). (c) Stereo picture of the model of the operator fragment.

2. MATERIALS AND METHODS

RNA runoff transcripts were prepared from linearised pSP64-BL (pSP64 plasmid containing a DNA sequence complementary to the b-loop of the translational repression region of MS2 RNA) essentially as described [10], labelled at the 3'-terminus [9] and the sequence confirmed by specific enzymatic digestion [11].

Modification of 3' labelled RNA with DEPC (Sigma) under 'native' conditions was performed in 200 μl structure buffer (see Fig. legends) containing ≈ 50,000 cpm 3'[^{32}P]RNA, 10 μg tRNA carrier (Sigma), and 5% (v/v) DEPC at 20°C for 60 min. Modification under denaturing conditions was performed in 200 μl D1 buffer (50 mM sodium cacodylate pH 6.8, 1 mM EDTA) containing ≈ 50,000 cpm 3'[^{32}P]RNA, 10 μg tRNA carrier and 0.5% (v/v) DEPC at 90°C for 10 min. All reactions were stopped by addition of 50 μl 1.5 M sodium acetate pH 6.0 and 750 μl ethanol. The recovered RNA pellets were then reprecipitated from 200 μl 0.3 M sodium acetate pH 6.0 and dried briefly under vacuum. Cleavage of RNA at the DEPC-modified sites with aniline (Sigma) was performed as described [9].

The oligonucleotides produced by treatment with aniline were separated on 20% (w/v) polyacrylamide/7 M urea gels [12]. The amount of radioactivity present in each band identified by autoradiography was quantitated by cutting the gel using the autoradiogram as a template and counting the slices by the Cerenkov effect in a liquid scintillation counter.

Filter binding assays were performed as described [3].

3. RESULTS

Fig. 2a shows the result of DEPC modification in different buffers of an SP6 RNA polymerase transcript en-

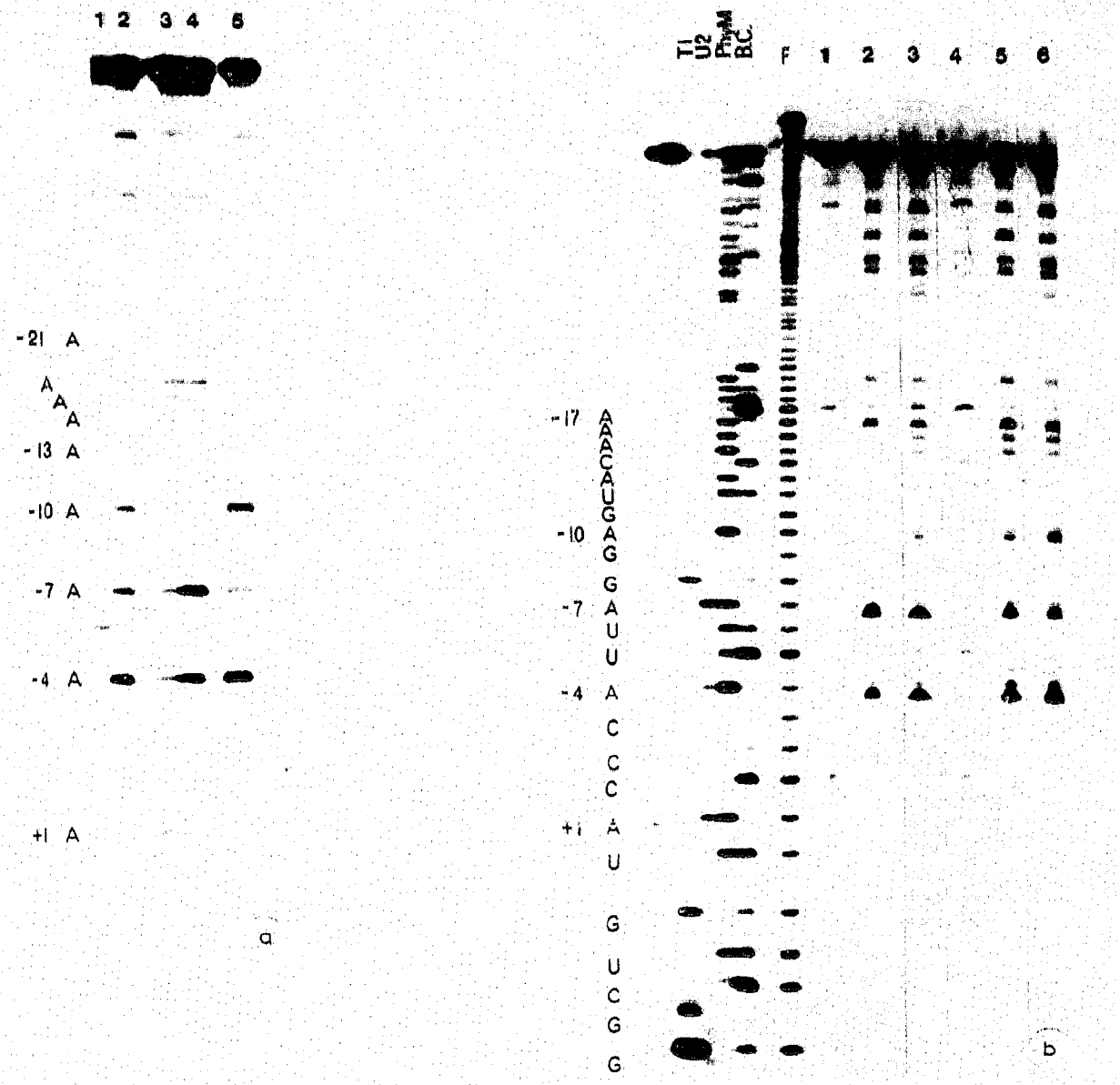

Fig. 2. (a) DEPC modification of b-loop RNA transcript under denaturing (lane 3), semi-denaturing (lane 4) and 'native' conditions (lanes 2 and 5). Modification conditions were: lane 2, 200 μl CMK buffer, 5.0% (v/v) DEPC for 60 min; lane 3, 200 μl D1 buffer, 0.5% (v/v) DEPC for 10 min at 90°C; lane 4, 200 μl D1 buffer, 5.0% (v/v) DEPC for 60 min; lane 5, 200 μl TMK buffer, 5.0% (v/v) DEPC for 60 min; lane 1 is an incubation control. All reactions were at 25°C unless otherwise stated. CMK buffer is 50 mM Na cacodylate pH 6.8, 10 mM $MgCl_2$, 80 mM KCl, 50 μg/ml tRNA. The SP6 polymerase product had the sequence; 5'rGAAUACAAGCUUGGGCUGCAGGUCGACUCUAGAGGAUCCCCGGC-CAUUCAAACAUGAGGAUUACCCAUGUCGAAUU-3', and this was confirmed by direct RNA sequencing. Secondary structure predictions of the transcript showed that only the desired b-loop structure would be stable. (b) Effect of varying pH and ionic strength on DEPC modification. Times and conditions of modification as in legend for Fig. 2a. Lanes 1 and 4 controls; 2, D1 (pH 6.8, I = 25 mM); 3, CKE (D1 + 150 mM KCl, I = 175 mM); 5, TE(50 mM Tris, pH 8.0, 1 mM EDTA); 6, TKE(TE + 150 mM KCl, I = 175 mM). Enzymatic sequencing lanes (T1, U2, PhyM and *B. cereus*) [11], together with a formamide ladder are shown alongside.

compassing the translational repression site. Modification at 90°C produces an even pattern of modification at all the A residues within the fragment as expected. Modification at 20°C produces a pattern of distinctly varying degrees of modification at different residues, confirming that the degree of modification is dependent on secondary and tertiary structure. A comparison of the rates of modification of A's in Watson-Crick base-pairs, those expected to be single-stranded and those in a neighbouring loop (data not shown) (Table I) shows that the three important adenine residues are the most extensively modified, i.e. are hyperreactive.

Variation of pH, ionic strength and divalent cation concentration resulted in essentially two distinct DEPC

Table 1

Nucleotide position	First order rate constants of DEPC modification (s^{-1}, $\times 10^{-4}$)		Surface accessibilities (Å²)			
	TMK	D1	Probe radius Å	1.4	2.8	5.6
A-21	1.0	2.9				
A-17	4.7	4.8				
A-16	1.6	0.73				
A-15	1.5	1.6				
A-13	no detectable modification					
A-10	9.6	1.5		7.0	2.0	0.0
A-7	2.3	12.0		15.0	14.0	3.0
A-4	7.0	7.5		16.0	15.0	14.0

The values for the first order rate constant are the linear regression slopes from plots of $\ln(1-P_n)$ vs. time where P_n is the probability of modification at base n, calculated from $P_n = R_n / \Sigma R_m\ (m > n)$. R_n is the amount of radioactivity in a band at position n. Surface accessibility calculations were performed on the model of the translational repression site for probes of three different radii and the results for the N7 atoms of the adenines in hyperreactive sites listed in square Ångstroms [6]. The largest probe size used predicts a relative reactivity between A-4 and A-7 of 4.6 close to the experimentally observed ratio of 3.4. However, it predicts zero reactivity at A-10 which is actually hyperreactive in these conditions suggesting that the true structure of this region is distinctly different from the model, which has the adenine intercalated between the neighbouring base pairs.

modification patterns (Fig. 2b). At low pH, ionic strength and divalent cation concentrations there is only modest modification at -10; -7 and -4 are strongly modified with -7 being approximately twice as reactive as -4. As the pH is raised from pH 6.8 to pH 8.0 and the ionic strength and divalent cation concentration are raised, the adenine at -10 becomes heavily modified and the relative degrees of modification at -7 and -4 are inverted.

Remarkably, pH has a very marked effect even though there are no obvious functional groups titrating in this pH range. A change of buffering ion from Tris to Hepes did not alter the results, and experiments in which the pH was raised in 0.2 pH unit steps suggested that the effect is gradual above pH 7.0. The results are consistent with the RNA adopting two major conformers which can be discriminated by their DEPC reactivity.

To investigate the role of the different conformers in coat protein recognition we carried out affinity and dissociation measurements using filter-binding assays, in either D1 or TMK; buffers which produce modification patterns representative of the two conformational extremes. The results are shown in Fig. 3. No difference could be detected in either the rate of dissociation or in the affinity of protein for RNA in either buffer. It is not possible to study the effect of bound coat protein on the DEPC modification because the reagent modifies the protein cysteine groups, one of which has been implicated in binding the RNA [13,14].

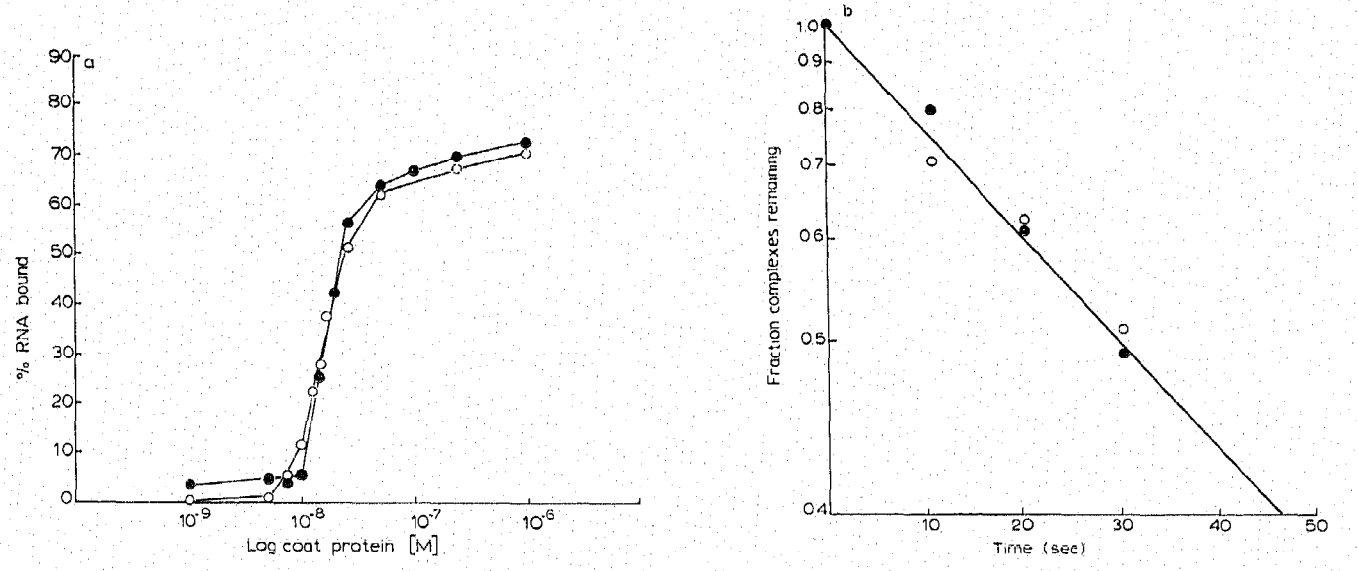

Fig. 3. (a) Coat protein excess binding curves for operator transcripts in TMK (●) and D1 (○) buffers. $K_d \approx 1 \times 10^8$ M in both buffers. (b) Dissociation kinetics of operator/coat protein complexes in the two buffers. Complexes were formed between 4 nM ^{32}P-radiolabelled RNA and 10 nM coat protein, dissociation initiated by the addition of 50 nM unlabelled operator and aliquots withdrawn for filter-binding. $k_{off} \approx 0.017$ s^{-1} in both TMK (●) and D1 (○).

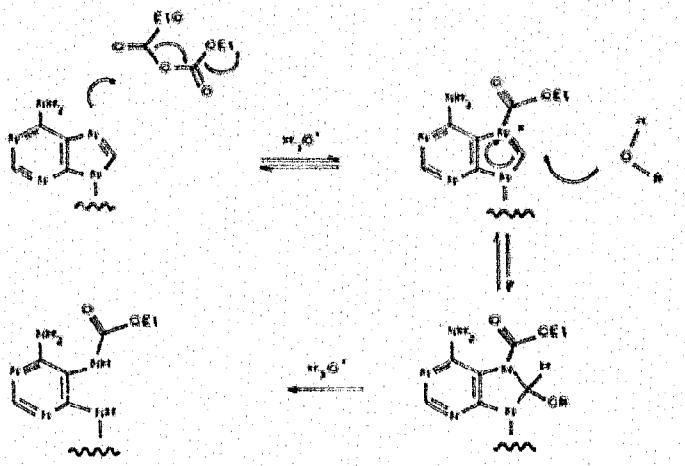

Fig. 4. Reaction scheme for the modification of adenine by DEPC.

4. DISCUSSION

DEPC modification of adenines is widely used as a structural probe of the conformation of RNA molecules. Lack of reactivity is usually taken as an indication that the adenine residue is base-paired and that stacking interactions inhibit reaction with DEPC. Our results with the MS2 translational operator suggest that this may be an oversimplification. Although it is clear that adenines in Watson-Crick base-pairs are essentially completely unreactive, adenines in other conformations can exhibit striking differences in the extent of reactivity.

Surface accessibility calculations on our model of the translational operator show that accessibility per se can not account for the observed hyperreactivity (Table I). However, the calculations do predict approximately the observed relative reactivity of A-7 and A-4 in TMK buffer. One possible explanation for the hyperreactivity comes from consideration of the reaction pathway of DEPC modification (Fig. 4). DEPC modification leads to opening of the five-membered ring and results finally in a carboxyethylated uridine derivative at the site of reaction [1]. The initial modification reaction is easily reversible and conversion to the final product depends on the availability of a suitable nucleophile, which commonly would be water. However, neighbouring bases in the stem-loop are close enough to the reactive adenines that they might supply the nucleophilic group required to lead to ring opening. Final cleavage with aniline would then reflect a combination of surface accessibility and nucleophile availability. A possible reaction scheme for one of the hyperreactive loop adenines is shown in Fig. 5. Changes in reactivity of the loop adenines in the different buffers may well represent true changes in accessibility, by say the binding of divalent cations. The very marked change in reactivity of the -10A from hyperreactivity to reaction rates similar to those which are single-stranded e.g. -15 and -16, may represent a distinct conformational change, perhaps by extrusion of the mismatched base from the stem. This idea suggests that only the intercalated conformation will be hyperreactive.

The reaction pathway outlined in Fig. 5 could be tested experimentally by observing the shift of the formyl group from the initially modified base to the neighbouring nucleophile. Further work is in hand to test this, to determine the accessibilities at adenine N1 positions and to try and model the conformational changes which take place.

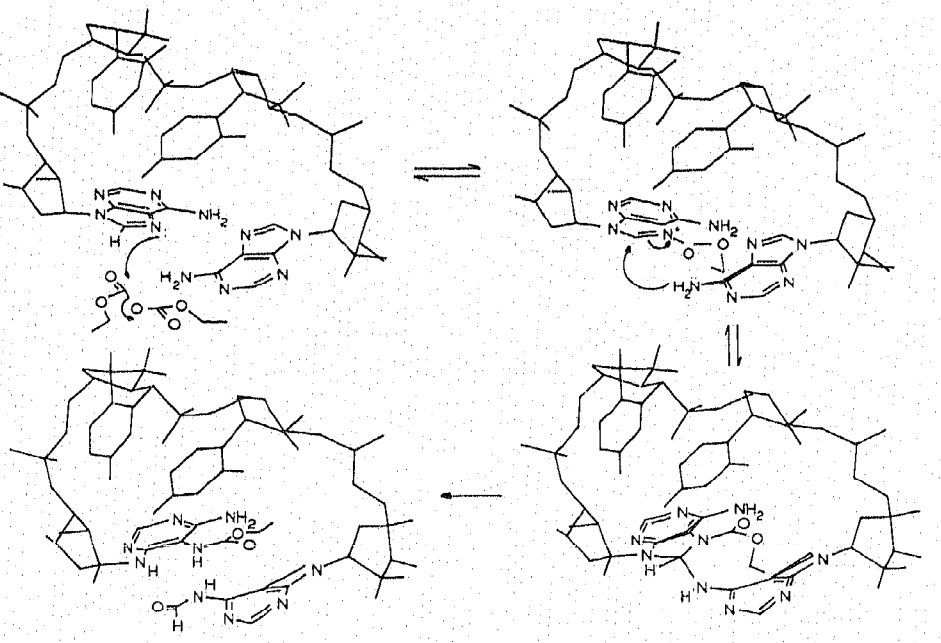

Fig. 5. Proposal for the possible involvement of a neighbouring base during modification of one of the loop adenines.

The apparent indifference of the coat protein to the conformer which is bound may indicate that interconversion is facile and that coat protein binding displaces an equilibrium, and/or that the reaction sequence has several steps in which the initial binding is not rate-limiting.

Our results suggest that for a simple RNA recognition site containing a single mismatched base (a) several conformations of such mismatched structures are energetically possible, and (b) that quantitative chemical modification of adenines by DEPC would seem to be a very sensitive probe of conformational changes.

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